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Mini-review

Application of combinatorial library methods in cancer research and drug discovery

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Summary: Combinatorial chemistry is now considered as one of the most important recent advances in medicinal chemistry. There are five general approaches in combinatorial peptide library methods: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. Except for the biological library approach, which is limited to peptide libraries with eukaryotic amino acids, all the other four synthetic approaches are applicable to peptide, non-peptide oligomer or small molecule libraries. Although non-peptide or small molecule libraries are generally prepared by a synthetic approach, recent advances in biosynthetic methods using enzymes may enable one to prepare chemical libraries that are otherwise difficult to synthesize chemically. In the 'one-bead one-compound' library method every member of the library is screened in parallel, but the chemical structure of the positive compound-bead has to be determined either directly or via an encoding strategy. A reliable high-throughput biological assay is needed for a successful combinatorial library screen. Solid-phase binding or functional assays as well as solution phase assays have been used successfully in various library methods. There has been enormous progress in the technological advances of molecular biology and the fundamental understanding of the molecular basis of cancer in recent years. By applying combinatorial chemistry and computational chemistry to the many cancer targets that have recently been identified, it is hopeful that more potent, more specific and less toxic anti-cancer agents will be developed in the foreseeable future. In addition to being a great tool for drug discovery, combinatorial chemistry has also proven to be invaluable in basic research. A few specific examples of the applications of combinatorial chemistry in basic cancer research and drug discovery are described in this mini-review.

Key words: cancer targets/combinatorial chemistry/drug discovery/peptide and chemical libraries

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Introduction

In traditional drug discovery, complex mixtures of natural products such as plant and animal extracts, fermentation broth or large collections of synthetic compounds are screened for a specific biological property. In the case of natural products, purification and identification of an active component often require several years of work. Several important anti-cancer agents currently being used in the clinic, such as taxol, bleomycin, vincristine, vinblastine, etoposide and doxorubicin, are in fact isolated from natural products. In the last few years an emerging technology known as combinatorial chemistry has shown great potential in speeding up the drug discovery process. Currently, virtually every major pharmaceutical company has incorporated or acquired this new technology in their drug discovery programs. Combinatorial chemistry is now considered as one of the most important recent advances in medicinal chemistry (Gallop *et al.*, 1994; Gordon *et al.*, 1994; Lam & Lebl, 1996; Thompson & Ellman, 1996). Not only can combinatorial library methods be used to generate new leads for a specific target; they can also be used as a

Table I Milestones in combinatorial chemistry

Year	*Milestone*	*References*
1984	Limited peptide library with the multi-pin technology	(Geysen *et al.*, 1984)
1985	Limited peptide library using the tea-bag technique	(Houghten, 1985)
1986	Iterative approach on solid phase peptide library screening using the multi-pin system	(Geysen *et al.*, 1986)
1986–90	Development of polynucleotide library methods	(Horwitz & Loeb, 1986; Oliphant *et al.*, 1986; Joyce, 1989; Blackwell & Weintraub, 1990; Ellington & Szostak, 1990; Pollock & Treisman, 1990)
1988	Introduction of the 'split synthesis' method on synthesizing a limited library of solution peptides	(Furka *et al.*, 1988a,b)
1990	Light-directed parallel peptide synthesis of a library of 1024 peptides on chip	(Fodor *et al.*, 1991)
1990	Successful application of the filamentous phage displayed peptide library method on a huge library of peptides	(Cwirla *et al.*, 1990; Devlin *et al.*, 1990; Scott & Smith, 1990)
1991	Introduction of the 'one-bead one-compound' concept, and successful application of this concept to a huge bead-bound peptide library	(Lam *et al.*, 1991)
1991	Successful application of the iterative approach on a huge solution phase peptide library	(Houghten *et al.*, 1991)
1992	Synthesis of a limited benzodiazepine-based small molecule library	(Bunin & Ellman, 1992)
1992–3	Development of encoding methods for the 'one-bead one-compound' non-peptide library	(Brenner & Lerner, 1992; Kerr *et al.*, 1993; Needels *et al.*, 1993; Nikolai *et al.*, 1993; Ohlmeyer *et al.*, 1993)

powerful alternative method to optimize the initial leads. Combinatorial library methods involve (i) the generation of a large number of compounds by randomly joining various building blocks together; (ii) a high throughput screening method by which the library can be screened for a specific biological property; and (iii) the identification of the active compound by a deconvolution method, by direct chemical analysis or by analysis of the coding structure. The milestones in the development of combinatorial chemistry are summarized in Table I.

Most medicinal chemists believe that a drug ought to be a small molecule as larger molecules often are neither orally active nor able to cross cellular membranes. Therefore, most pharmaceutical companies have focused their efforts on developing small molecule combinatorial chemistry. Although orally active drugs are, in general, more useful, one should not forget that almost all anti-cancer chemotherapeutic agents given today are administered i.v. even though some of these drugs are available in oral form. For some extracellular targets, larger ligands derived from combinatorial peptide libraries, polynucleotide libraries or other synthetic oligomeric libraries may sometimes be preferable. For instance, a synthetic gonadotrophin-releasing hormone analog, goserelin, is currently being used in the clinic as one of the most important anti-prostate cancer agents. Goserelin is a synthetic decapeptide (mol. wt 1269) administered s.c. in a depot form with continuous release over a 28 day period. Compliance with this drug is not a problem as it is administered in the clinic once a month and is probably preferable to an oral form with a multiple dosing schedule each day. Various types of combinatorial libraries are summarized in Figure 1. In

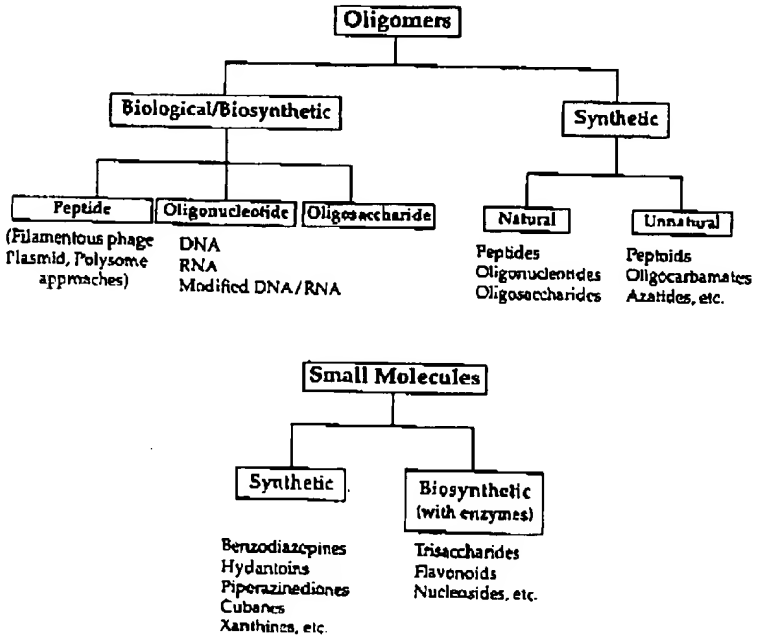

Figure 1 Different types of combinatorial libraries.

Table II Combinatorial peptide library methods[a]

1.	Biological libraries
	Filamentous phage
	Plasmid
	Polysome
2.	Spatially addressable parallel solid phase or solution phase library
	Multi-pin technology
	SPOTs-membrane
	Light-directed peptide synthesis on chips
	Diversomer technology
3.	Synthetic library methods requiring deconvolution
	Iterative approach
	Positional scanning
	Recursive deconvolution
	Orthogonal partition approach
4.	'One-bead one-compound' library method
5.	Synthetic library method using affinity chromatography selection

[a]Except for the biological library approach, which is limited to peptide libraries with eukaryotic amino acids, all the other four synthetic approaches are applicable to peptide, non-peptide oligomer or small molecule libraries.

this mini-review I do not attempt to give a comprehensive review on the subject. Instead, I would like to give an overview on the various approaches of the combinatorial library methods with emphasis on peptide, non-peptidic oligomeric and small organic libraries, and give a few specific examples on the applications of combinatorial library methods in basic cancer research and drug discovery.

Peptide libraries

As indicated in Table I, the combinatorial library field was created when the first limited peptide library was synthesized (Geysen *et al.*, 1984). Initially, only hundreds to a few thousands of peptides could be synthesized and screened. In 1990 and 1991, three totally different combinatorial library methods were introduced: (i) the filamentous phage displayed peptide library method (Cwirla *et al.*, 1990; Devlin *et al.*, 1990; Scott & Smith, 1990); (ii) iterative approach to a solution phase peptide library (Houghten *et al.*, 1991); and (iii) the 'one-bead one-compound' combinatorial peptide library method (Lam *et al.*, 1991). These methods enable one to rapidly prepare and screen millions of random peptides. Since then, the field of synthetic combinatorial chemistry has exploded as many of the underlying principles and methods of the synthetic combinatorial peptide library methods can readily be applied to small molecule libraries. Currently, there are five general approaches in combinatorial peptide library methods (Table II) (Lam, 1995, 1996).

Biological libraries

In 1988 Parmley & Smith proposed the use of filamentous phage to display a random oligopeptide on the amino terminus of the viral pIII coat protein. This was accomplished by

the insertion of a stretch of random deoxyoligonucleotide into the pIII gene of filamentous phage. In 1990 three groups reported the successful application of this approach in generating millions of random peptides from which specific ligands against monoclonal antibodies (Cwirla *et al.*, 1990; Scott & Smith, 1990) or streptavidin (Devlin *et al.*, 1990) were isolated. Since then, numerous groups have applied these techniques in identifying peptides for various molecular targets. Subsequently, related techniques on expressing peptides in libraries of plasmids (Schatz, 1993) and polysomes (Kawasaki, 1991) were developed. The major advantage of the biologic approach is that the size of the grafted peptides is not limited by the constraints of synthetic peptide chemistry. Furthermore, the biological approach enables one to take advantage of known protein folds (e.g. immunoglobulin fold or conotoxin fold) by grafting random oligopeptides on such tertiary folds. However, the biological approach in general is limited to the 20 eukaryotic amino acids only, and incorporation of unnatural amino acids or other organic moieties into these libraries is not feasible. Additionally, the screening assays of the biological libraries are generally limited to binding assays (e.g. panning) and not applicable to most of the solution phase assays utilized in the standard drug screen.

Spatially addressable parallel solid phase or solution phase library

In this method the peptides are synthesized on solid phase support in a spatially addressable format and the amino acid sequence of each of the peptides is predetermined. The synthesis and screening of these libraries are performed in parallel. Depending on the method used, screening can be performed while the peptides are still attached to the solid support (e.g. a binding assay), or the peptides can be released from the solid support for solution phase assay. The positive peptide can be localized and therefore identified. The advantage of this approach is that the chemical structure of the peptides is predetermined, and therefore subsequent structure determination is not needed. However, the major limitation of this method is that only a limited number of peptides can be synthesized in parallel and the library is relatively small. The three techniques based on this strategy are as follows.

Multi-pin technology. In this method, Geysen *et al.* (1984) grafted acrylic acid onto polyethylene pins by gamma irradiation. Biological screening was performed by an enzyme-linked immunosorbant assay (ELISA) on the peptide pin. Later, cleavable linkers were developed (Maeji *et al.*, 1990) so that peptides could be released from the pin for solution assay.

SPOTS membrane. Frank (1992) essentially followed Geysen's strategy except that a cellulose membrane or paper was used, rather than the polyethylene pins, as the solid support for peptide synthesis.

Light-directed peptide synthesis on chips. Fodor *et al.* (1991) synthesized minute quantities of 1024 peptides on a single glass plate by using a photolithographic masking process with a photolabile Nα-amino protecting group. Each peptide occupies a 50 × 50 mm area. Because of the limited quantity of each peptide available, biological assay is restricted primarily to binding, quantitated by fluorescent microscopy. This method requires complicated instrumentation and is not widely available. Furthermore, the practical number of peptides one can generate with this method is probably limited (e.g. no more than 100 000).

The 'diversomer technology' first described by DeWitt *et al.* (1993) and applied to

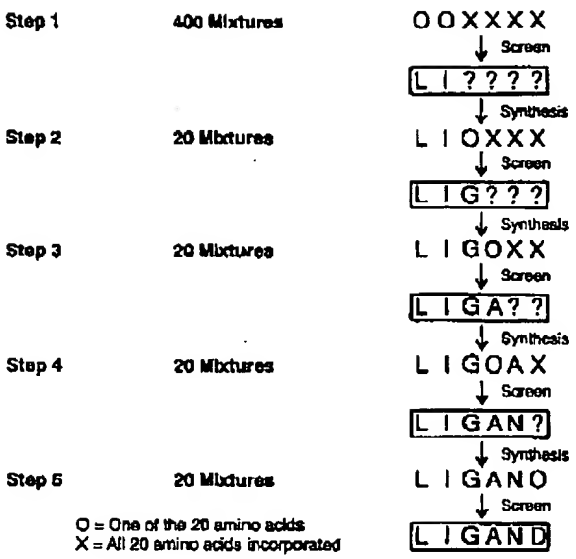

Figure 2 Scheme of the iterative approach for combinatorial library method that requires deconvolution. (Adapted from *Encyclopedia of Molecular Biology and Biotechnology*, p 520. © 1996 VCH, John Wiley.)

nonpeptide, nonoligomeric chemical libraries is another variation of the spatially addressable parallel library approach and certainly can also be applied to peptide libraries.

Synthetic library methods requiring deconvolution

These methods involve the synthesis of a number of peptide mixtures. The composition of peptides within each mixture is known. These peptide mixtures are then screened for a specific biological property. From the results of the biologic assays, one may be able to deduce the structure of the active component. In these methods, isolation of the active component for structural determination is not needed. The four general deconvolution approaches are: (i) iterative approach (Geysen *et al.*, 1986; Houghten, 1991); (ii) positional scanning approach (Dooley & Houghten, 1993); (iii) recursive deconvolution strategy (Erb *et al.*, 1994); and (iv) the orthogonal partition approach (Deprez *et al.*, 1995; Pirrung & Chen, 1995).

The iterative approach was first successfully applied by Geysen *et al.* (1986) in the multi-pin system in an on-pin ELISA assay to define a mimotope for a monoclonal antibody directed against a discontinuous epitope on foot-and-mouth disease virus. Later, Houghten *et al.* applied the same iterative strategy in a solution phase assay for various monoclonal antibodies (Houghten, 1991), opiate receptor (Houghten *et al.*, 1992) and anti-bacterial activity (Blondelle *et al.*, 1995). The overall scheme for the iterative approach is summarized in Figure 2. In this method, multi-step synthesis and analysis are required for deconvolution. For example, in a random hexapeptide library, one of the 20 amino acids are incorporated

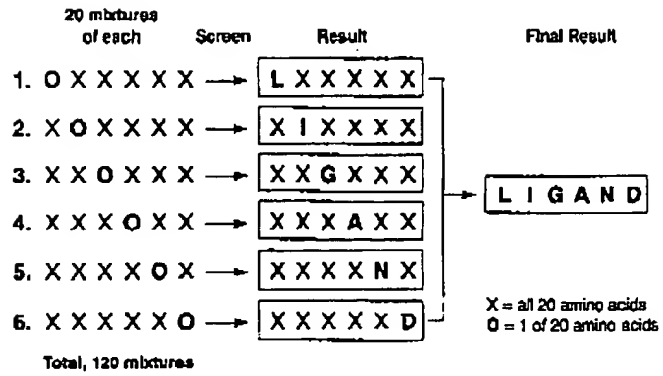

Figure 3 Scheme of the positional scanning approach for combinatorial library method that requires deconvolution.

into the first two residues while all 20 amino acids are incorporated into the remaining four residues, i.e. randomization (step 1). As a result, 400 mixtures of peptide are generated. These mixtures are then screened against a biological function and the mixture with the highest activity is identified. After the first two residues are defined, these two residues are fixed and one of the 20 amino acids are then incorporated into the third residue while the remaining three residues are randomized (step 2). This results in the generation of 20 peptide mixtures, which are then screened for biological activity. The third 'active' residue is then defined and the same iterative process is repeated for three more cycles until the last 'active' residue is identified.

Dooley & Houghten (1993) later introduced a related approach called the 'positional scanning method' (Figure 3). In this method, 120 mixtures of hexapeptides are synthesized with one of the 20 amino acids incorporated into one specific residue while all 20 amino acids are incorporated into the remaining residues (randomization). The 120 mixtures are then subjected to biological testing. From the results of the 120 assays, one can then define each 'active' residue. This method is much more efficient than the iterative approach as it is a one-step approach and multi-step synthesis and analysis is not needed. However, this method assumes that the contribution of each residue to the biological activity is independent of each other. This approach works very well if there is only one predominant motif. If there are multiple motifs, one risks the possibility that the results will be scrambled and uninterpretable.

Erb *et al.* (1994) described a recursive deconvolution strategy in which a 'split synthesis' method (see below) is used to generate the library. In each synthetic step, a portion of the resin from each reaction vial is set aside and catalogued. The set-aside resins are then used for the synthesis of sublibraries at each iterative step. This strategy facilitates the iterative approach described above.

Two groups (Deprez *et al.*, 1995; Pirrung & Chen, 1995) reported the orthogonal partition approach of deconvolution by synthesizing and screening multiple orthogonal sublibraries. In this method, multiple sublibraries are synthesized. The composition of the subunits of

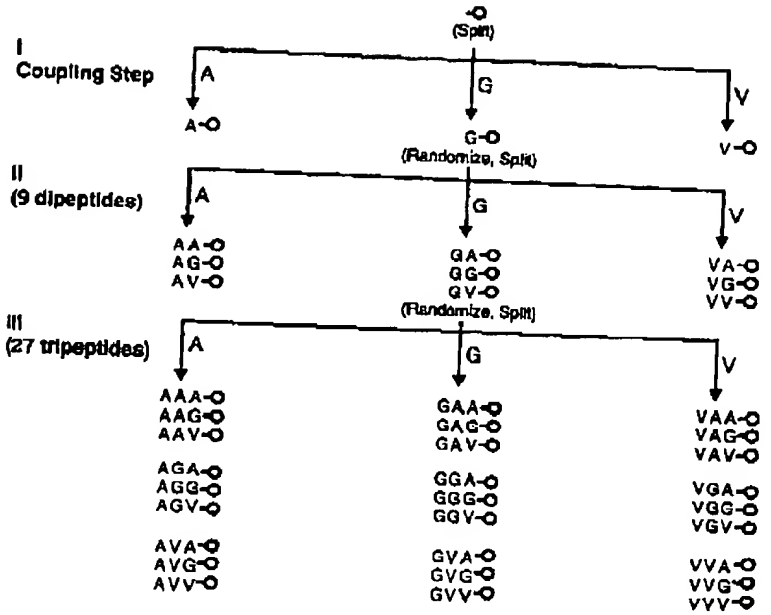

Figure 4 Scheme of the 'split synthesis' method to generate a 'one-bead one-compound' combinatorial library. (Adapted with permission from *Nature*, 354, 82. © 1991 MacMillan Magazines.)

each sublibrary are segregated in an orthogonal fashion so that the 'active' residue can be deduced, in the form of a matrix, by the synthetic history of each of the sublibraries. Similar to the positional scanning approach, if there are multiple active motifs, the result may be difficult to interpret.

'One-bead one-compound' library method

In this approach the peptide library is generated by a solid phase technique (Merriefield, 1963) using a 'split synthesis' method (Furka *et al.*, 1991; Houghten *et al.*, 1991; Lam *et al.*, 1991). In the 'split synthesis' (Figure 4) the resin beads are first divided into several (e.g. 20) equal aliquots and each of the 20 amino acids are added into each reaction vessel and the reaction is driven to completion. The resins are then thoroughly mixed, deprotected and partitioned into 20 aliquots again for the next coupling cycle. The process is repeated several times until a peptide with a desired length is obtained. Since each resin bead encounters only one amino acid at each coupling cycle, and the reaction is driven to completion, the end result is that every peptide ($\sim 10^{13}$ copies for a 100 μm bead) on each individual bead is unique and identical. A pentapeptide library with 20 amino acids incorporated into each coupling cycle will have 20^5 or 3.2 million possible permutations. The synthesis of such a library can generally be completed in 2–3 days. This 'one-bead one-compound' concept was first described by Lam *et al.* in 1991. Although Furka *et al.* first presented a brief account of

the 'split synthesis' method for the preparation of a very small library (a total of 27 peptides) of solution peptides in two abstracts (Furka *et al.*, 1988a,b), they failed to recognize (Furka *et al.*, 1991) the 'one-bead one-compound' concept and its importance in the screening strategy (Lam *et al.*, 1991; Lam & Salmon, 1996).

Lam *et al.* (1991) applied the 'one-bead one-compound' combinatorial peptide library method to synthesize and screen a random peptide bead library of over a million and was able to identify peptide ligands that bind to an anti-β-endorphin monoclonal antibody or streptavidin. An enzyme-linked colorimetric assay was used to screen the peptide bead library. In this assay, peptide beads are incubated with receptor–alkaline phosphatase complex. The library is then washed thoroughly and a standard substrate such as 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) is added to the bead library. Beads that interact with the receptor–alkaline phosphatase complex turn turquoise in color (Lam & Lebl, 1994). The color bead is then physically isolated under a dissecting microscope with the aid of a micropipette, transferred onto a glass filter and inserted into a cartridge for automatic microsequencing. In the last few years this colorimetric method has been applied successfully in the author's laboratory to identify specific ligands for various macromolecular targets such as monoclonal antibodies (Lam *et al.*, 1991), lymphoma surface idiotypes (Lam *et al.*, 1995a), MHC class I molecules (Smith *et al.*, 1994), streptavidin (Lam *et al.*, 1991; Lam & Lebl, 1992) and avidin (Lam & Lebl, 1992). Besides on-bead binding assays as described above, the 'one-bead one-compound' concept can also be applied to other on-bead functional assay systems as well as solution phase assay systems (see the section on screening strategies below). For a recent review on the 'one-bead one-compound' combinatorial library method, see Lam & Lebl (1996) and Lam *et al.* (1997).

Synthetic library method using affinity chromatography selection

In this method the peptide library is first synthesized in solid phase, usually by a 'split synthesis' method to obtain an equimolar mixture of random peptides. The peptides are then cleaved off the resins to form a solution phase peptide library. The peptide library is then loaded onto an affinity chromatography to select for peptides that bind to the immobilized receptor on the affinity column. After thorough washing, the bound peptides are eluted for structure determination. Ideally, the eluted peptides should be further fractionated to obtain individual purified peptide prior to microsequencing. Non-specific binding may become a major problem if the affinity between the ligand and receptor is not very high (e.g. >1 μM), or if the number of different peptides in the library is huge (e.g. 10^6–10^8). Additionally, enough purified peptide (e.g. >1 pmol) must be retrieved for accurate microsequencing. As an alternative to microsequencing individual purified peptides, one may elect to sequence all the eluted peptides collectively without fractionation. However, the interpretation of the microsequencing data may be difficult because of the high background. If there is more than one predominant motif in the mixture, the result will be uninterpretable. As a proof of concept, Zuckermann *et al.* (1992) applied the affinity selection method to isolate three peptides from a very small peptide library (19 components) that bind to an anti-gp120 monoclonal antibody. More recently, Cantley's group reported the successful application of the affinity selection method to identify peptide motifs for SH_2 domains (Songyang *et al.*, 1993) and kinase domains of protein tyrosine kinases (Songyang *et al.*, 1995) from huge peptide libraries. Details of this work will be discussed below.

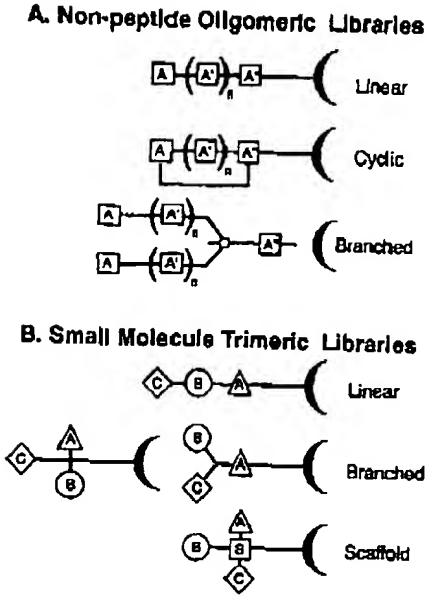

Figure 5 Various forms of assembly of synthetic oligomeric and small molecule libraries.

Non-peptide oligomeric and small molecule non-oligomeric libraries

Chemistry

Within two years after the publications by Lam *et al.* (1991) and Houghten *et al.* (1991) on the successful application of two different synthetic combinatorial peptide library methods to identify ligands from huge libraries of peptides (>10^6) for specific macromolecular targets, there was an explosion of literature on synthetic combinatorial chemistry, particularly in the area of non-peptide and small molecule libraries (Thompson & Ellman, 1996). Additionally, with the advances of methods in enzyme-catalyzed reactions, some non-peptide or small molecule libraries can, in principle, be synthesized by biosynthetic approaches. As mentioned earlier, the general principles for the synthetic peptide library methods are readily applicable to the non-peptide oligomeric or small molecule libraries. Figure 5 summarizes the various forms of assembly of synthetic libraries. Non-peptide oligomeric libraries include peptoids (Simon *et al.*, 1992), oligocarbamate (Cho *et al.*, 1993), oligoureas (Burgess *et al.*, 1995), vinylogous sulfonyl peptides (Gennari *et al.*, 1995), peptidosulfonamides (duBont *et al.*, 1996), azatides (Han & Janda, 1996) and ketides (Reggelin and Brenig, 1996, Khosla and Zawada, 1996). These libraries, similar to peptide libraries, can be linear and highly flexible or can be constrained by, for example, cyclization (Figure 5A). It can also be branched. The main advantage of these non-peptide oligomeric libraries is that they are resistant to proteolytic enzymes and therefore may be more

therapeutically useful. Additionally, these oligomers may exhibit specific physical, chemical or structural properties that are more favorable therapeutically.

The main advantage of small molecule non-oligomeric libraries in the drug discovery process is that drug leads discovered from such screens are small molecules and may already contain lipophilic, aromatic or heterocyclic structures that allow them to cross cell membranes, making them ideal candidates for drug development. Many of the orally active drugs used today have a mol. wt betwen 300 and 600. A trimeric library would be ideal for this molecular weight range. In a trimeric small molecule, one hundred subunits per coupling cycle will generate a library of 100^3 or 1 million unique compounds. Figure 5B illustrates four general ways of building a trimeric small molecule library in solid phase: a linear form, two branched forms and a scaffolded form. Many of the strategies used today rely on development of new solid phase chemistries for the coupling of functional subunits that are readily available commercially. The chemistry of small molecule combinatorial library methods has recently been reviewed (Thompson & Ellman, 1996) and I shall not attempt to duplicate it here. Examples of the small molecule non-oligomeric libraries include derivatives of 1,4-benzodiazepin-2-ones, 1,4-benzodiazepine-2,5-diones, diketo-piperazines isoquinolinones, 1,4-dihydropyridines, dihydro- and tetrahydroisoquinolines, pyrrolidines, hydantoins, thiohydantoins, thiazolidine-4-carboxylic acids, 4-thiazolidinones, imidazoles, triazines, acylpiperidines, β-lactams, tetrahydrofurans, benzopyranes, isoxazole, triazoles, cyclopentane, cyclohexane, quinazoline and carboline. I predict that in the next few years many new efficient coupling chemistries in solid phase will be developed (Früchtel & Jung, 1996). In addition, a series of novel functionalized subunits specifically designed for small molecule combinatorial chemistry will also become commercially available.

Another method of generating combinatorial chemical libraries is to use biosynthetic approaches or 'combinatorial biocatalysis'. In this approach the coupling reactions are catalyzed by enzymes. For instance, a polyketide library (Khosla & Zawada, 1996) and a small library of taxol derivatives (Khmelnitsky *et al.*, 1996) were produced by this approach.

Structure determination

For the library methods that require deconvolution (see above), structure determination by physicochemical methods is not necessary because the chemical structure of the active compound can be deduced from the synthetic history and assay results of the mixtures of compounds. Similarly, in the spatially addressable library methods the structure of each compound in the library is already known; therefore, structure determination of the positive lead is not needed. However, in the 'one-bead one-compound' combinatorial library method the chemical structure of compounds on individual bead is not known and therefore an individual positive bead has to be isolated for structure determination. In peptide libraries automatic microsequencing by Edman degradation is the method of choice since current technology enables detection down to 1 pmol of peptide. A typical 100 μm bead contains ~100 pmol of peptides. Since microsquencing by Edman degradation is not suitable for non-peptide oligomers or small organic molecules, one needs to use alternative approaches. If the number of compounds in a library is small or if one of the subunits in the library is known (e.g. the beads of the last coupling cycle remained segregated during the screening), mass spectroscopy alone may be sufficient for structure determinati n of the active ligand. Alternatively, Youngquist *et al.* (1994) used a limited capping technique to partially cap each

coupling cycle, and they were able to determine the structure of peptides by matrix-assisted laser desorption ionization mass spectrosopy. In principle, this limited capping method can also be applied to nonpeptide oligomeric or small organic molecule libraries. A similar approach has also been described by Sepetov *et al.* (1995).

Another approach is to apply an encoding strategy to tag each chemical subunit during the 'split synthesis', then the chemical structure of the positive ligand can be deduced by analyzing the structure of the tag. Various tags have been used successfully in the encoding strategies. These include polydeoxynucleotide (Brenner & Lerner, 1992; Needels *et al.*, 1993), peptide (Kerr *et al.*, 1993; Nikolaiev *et al.*, 1993) and haloaromatic derivatives (Ohlmeyer *et al.*, 1993; Nestler *et al.*, 1994). A tag corresponding to each subunit of the testing ligand is incorporated into the beads during the 'split synthesis' process. For every coupling cycle, tags are coupled immediately after coupling of the ligand subunits but before the mixing of the resins. In some cases it may be advantageous to spatially segregate the testing ligand [e.g. on the surface of the bead from the coding sequence (e.g. inside the bead)] (Vagner *et al.*, 1996). The polydeoxynucleotide tag can be determined by polymerase chain reaction followed by DNA sequencing; the peptide tag can be determined by automatic microsequencing with Edman degradation; and the haloaromatic tag can be determined by electron capture gas chromatography. There are two general coding systems: the sequential coding system and the digital or binary coding system. In the sequential coding system the tag is connected to the bead as a linear oligomer and the oligomeric coding sequence determines the sequence or structure of the testing ligand. This is the principle behind the polydeoxynucleotide tagging (Needels *et al.*, 1993) and some of the peptide tagging systems (Kerr *et al.*, 1993; Nikolaiev *et al.*, 1993). The tags of the digital or binary coding system, on the other hand, do not rely on the sequence of the tagging subunits. Instead, all the tags are cleaved from a bead and analyzed simultaneously. The composition of the tag, in a binary fashion, allows one to determine the chemical nature as well as the position of each subunit (i.e. residue 1, 2 or 3) of the testing ligand. Two groups have reported using this binary tagging approach to facilitate the coding/decoding process: Nestler *et al.* (1994) used the haloaromatic tags and Lebl *et al.* (1995) used a series of specially modified amino acids as the tags.

Recently, two groups reported the use of radiofrequency transponders for library encoding (Moran *et al.*, 1995; Nicolaou *et al.*, 1995). However, because of the large physical size of the current generation of radiofrequency transponders, this tagging technique can only be applied to very large beads (e.g. 8 mm diameter) or individual packages of beads.

Screening strategies

A reliable high-throughput biological assay is needed for a successful combinatorial library screen. Although many standard 96-well plate solution phase biological assays are available, several new assay systems specifically designed for combinatorial chemistry have been developed. The choice of assay system depends on the availability of specific reagents and resources, the nature of the biologic targets, the expertise of the researcher, and the type of combinatorial library method one decides to use. In general, the assay systems can be catagorized into: solid phase assays, in which the ligands are still covalently attached on the solid support, or solution phase assays, in which the ligands have to be released from the solid support in order to elicit a biological response. Except for the biological libraries,

the following discussion applies to both peptide and non-peptide combinatorial library methods.

Solid phase assay

In this assay system the ligands are still covalently attached to the solid support. The solid support could be a polyethylene pin (Geysen *et al.*, 1984), a bead (Lam *et al.*, 1991), a cellulose sheet (Frank, 1992), glass (Fodor *et al.*, 1991) or a filamentous phage (Scott & Smith, 1990). The assays could involve (i) direct binding of target macromolecule or intact cell to the ligand on the solid support, or (ii) detection of functional properties such as proteolytic or phosphorylation substrates (Lam & Wu, 1994; Meldal *et al.*, 1994).

Binding assay. In the case of synthetic library methods, the binding of the targets to the ligand on the solid support can be detected either directly by visualization [e.g. a color target such as a dye (Lam *et al.*, 1994; Wennemers & Still, 1994)] or indirectly by using a target covalently attached to a reporter group such as an enzyme (Lam *et al.*, 1991), a radionuclide (Kassarjian *et al.*, 1993), a fluorescent probe (Yu *et al.*, 1994) or a color dye (Ohlmeyer, 1993). The enzyme-linked colorimetric assay that was first employed in the 'one-bead one-compound' combinatorial library method (Lam *et al.*, 1991) is simple and highly efficient. In order to minimize the false positive beads, a dual-color detection scheme was developed (Lam *et al.*, 1995b). Alternatively, the positive beads can be decolorized with dimethylformamide and reprobed either in the presence of a competing ligand or with a different secondary antibody (Lam & Lebl, 1996). Ohlmeyer *et al.* (1993) used a dye-labeled target to screen peptide bead libraries. The same group also designed a dual-color screening method using two different color dyes to detect specific binding (Boyce *et al.*, 1994). The main problem for dye-labeled targets is that dye alone tends to bind to many ligands in a library. Therefore this often complicates the screening process. Fluorescent labeled receptor (Needels *et al.*, 1993) is useful particularly if a fluorescence-activated cell sorter is used in the selection of the positive beads.

Besides macromolecular targets, one can also screen for cellular binding using intact cells. With this method, peptides that bind to the surface integrin of a prostate cancer cell line have been identified (Pennington *et al.*, 1996).

Functional assay. Although most functional assays traditionally are performed in solution phase (e.g. using a 96-well plate format), specific functional assays have been developed for detecting ligands that are covalently attached to a solid support. Lam and co-workers (Lam & Wu, 1994; Wu *et al.*, 1994) used a 'one-bead one-compound' library method to identify peptide substrate motifs for protein kinases. In this assay a random peptide bead library is incubated with [γ-^{32}P]ATP and a protein kinase. After thorough washing, the beads are then immobilized on a glass plate with molten 0.5% agarose. After drying, the immobilized beads are exposed to an X-ray film. The autoradiogram is then used to localize and isolate the beads for microsequencing. Using this screening assay, the substrate motifs for cAMP-dependent protein kinase (Wu *et al.*, 1994) and p60$^{c\text{-}src}$ protein tyrosine kinase (PTK) have been identified (Lam *et al.*, 1995c). Similar methodology has also been applied to the SPOTS membrane libary for the identification of peptide substrate motif for cAMP-dependent and cGMP-dependent protein kinases (Tegge *et al.*, 1995). In principle, in addition to protein phosphorylation, these functional screening methods can also be applied to many other different post-translational modification reactions such as farnesylation and glycosylation.

Meldal *et al.* (1994) reported a novel fluorogenic quenching screening method to identify a proteolytic substrate motif from a 'one-bead one-compound' library. In this assay a fluorescent molecule is built into the carboxyl end of a random peptide library and a fluorescent quenching molecule is attached at the amino terminus. The bead library is then incubated with specific protease. The peptide on the bead with the appropriate substrate motif will be cleaved, releasing the quencher and resulting in an increase in fluorescence of the positive bead. A related approach to directly screen for protease inhibitor has also been developed (Meldal & Svendsen, 1995).

Solution phase assay

Traditionally, solution phase assay is the standard assay system for most drug screening efforts. The compound, whether it is synthetic or derived from a fermentation broth, is added in a soluble form to the assay system. Numerous solution phase assay systems are available, e.g. competitive receptor binding assay with a known radiolabeled ligand, competitive ELISA assay using plate-coated antigens, enzymatic assays such as a proteolytic assay using a fluorogenic substrate, anti-bacterial and anti-cancer assays using intact cells, or other cell-based signal transduction assays. Many of these assays have been adapted to a 96-well format and can certainly be used to screen combinatorial libraries. Because the number of compounds or mixtures of compounds generated by combinatorial chemistry is enormous, the current trend is to improve the efficiency of the high-throughput screen by miniaturizing as well as automating these solution phase assays. Solution phase assays have been the main screening method for most combinatorial library methods that require deconvolution.

Most of the published literature on the 'one-bead one-compound' library method uses solid phase on-bead screening assays. However, as indicated in the initial publication (Lam *et al.*, 1991; Lam & Salmon, 1992), solution phase assays can also be used for this library method. Single or double-orthogonal cleavable linkers (Lebl *et al.*, 1994) can be incorporated into the preparation of the library so that compounds can be released from the beads for biological testing (Salmon *et al.*, 1993, 1996; Jayawickreme *et al.*, 1994).

The choice of specific assay systems largely depends on the nature of the biologic target and the type of combinatorial library method one uses. An additional factor includes the availability of specific reagents such as purified receptors, enzymes, antibodies or radiolabeled ligands. In general, solid phase binding assays, when compared with solution phase assays, are much more efficient. However, purified targets are often required for solid phase binding assays. Sometimes it is advantageous to combine both solid phase and solution phase assays during the screen. For example, in the 'one-bead one-compound' library method, beads that are positive in the solid phase assay (e.g. enzyme-linked colorimetric assay) can be isolated and subjected to releasable solution phase assay for confirmation of biological activity. In this way, the screening process becomes much more efficient and the positive leads isolated are more likely to be true positives.

Cancer targets

Although there has been enormous progress in the technological advances of molecular biology and the fundamental understanding of the molecular basis of cancer in recent years (Mendelsohn *et al.*, 1995), translation of such knowledge to the development of useful

therapies for cancer is not an easy task. Currently, numerous potential cancer targets have been identified and can be readily cloned, expressed and obtained in purified forms. Genetic manipulation enables one to develop new cell lines and therefore new assay systems for many cancer targets. Relying on traditional drug discovery methods for these many targets would be very difficult. However, the advent of combinatorial library methods in the last few years may greatly facilitate such endeavors.

A commonly asked question is: 'Which combinatorial library method is better?' There is no simple answer to this question as each of the methods described above has its advantages and disadvantages. One should tailor a method to a specific project. Before choosing a specific combinatorial library method or a specific assay system one needs to first consider the kind of drug one wants to develop. Should it be a small molecule or should it be a slightly larger molecule? Is parenteral administration acceptable? As indicated earlier, this is often the case for anti-cancer drugs. Is the cancer target intracellular or extracellular? What are the chances that a small molecule will be effective for that target? In my opinion, a small molecule combinatorial library method is the method of choice for intracellular targets, because drug leads discovered from a small molecule library have a much better chance of being able to penetrate cell membranes. A small molecule library is also ideal for the development of enzyme inhibitors since enzyme active sites are often relatively small and there is a good chance that potent inhibitors can be discovered from such a library. For extracellular targets or receptors that normally bind to a larger ligand (e.g. cytokines, insulin), a small molecule drug of mol. wt 500 may not be able to bind to the receptor with sufficient affinity to be clinically useful. In this case a peptide library, an oligomeric non-peptide library or a non-peptide library with a large scaffold may be preferable. These drugs may be administered parenterally. As for the development of tumor vaccine, a peptide or glycopeptide library instead of a small molecule library would be the method of choice.

Inasmuch as a small molecule drug is preferable for intracellular targets, peptide libraries are sometimes very useful for the development of a drug lead. Based on the information from the peptide lead, one may then design peptidomimetic libraries to further optimize that lead. This strategy is probably useful for the development of inhibitors for enzymes such as protein kinases and proteases that catalyze peptide substrates. For example, rather than directly screening a random small molecule library for $p60^{c\text{-}src}$ protein tyrosine kinase, the substrate motifs of this enzyme can be determined using a random peptide library approach (Lam *et al.*, 1995c; Lou *et al.*, 1996). Based on the peptide motif, one can then design peptidomimetic libraries and screen for $p60^{c\text{-}src}$ PTK inhibitory activities. With this strategy, one can focus on the development of inhibitors against the peptide substrate pocket rather than the ATP binding pocket. I believe that more selective PTK inhibitors can be developed with this approach.

With the availability of numerous cancer targets, many researchers have focused their drug development efforts in discovering compounds against specific targets. However, one should not forget that many of the anti-cancer agents, except for antimetabolites, currently used in the clinic today were discovered by cell-based cytotoxicity assay without any specific cancer target in mind. Currently the National Cancer Institute is conducting *in vitro* screening with several different kinds of human cancer cell lines (including many solid-tumor lines) for the development of new anticancer agents as well as profiling of known anti-cancer drugs (Boyd & Paull, 1995). Very little work has been done in this area using combinatorial library approaches. Recent application of the 'one-bead

one-compound' combinatorial library method in random screening for anti-cancer activity using a cell base cytotoxicity assay (Salmon *et al.*, 1996) will be given below.

Selected examples of applications in the cancer field

Although there are several reports on the application of combinatorial library methods in basic cancer research, there have been very few reports in the literature on using combinatorial library methods in cancer drug development. However, I anticipate that in the next few years there will be more publications on this subject as more cancer researchers are now using this technology. Targeting the signaling pathway has been considered by many investigators as a fruitful area in cancer drug development. Protein tyrosine kinases (PTKs), being main players in the signal transduction pathway, have therefore drawn enormous attention (Burke, 1994; Powis & Workman, 1994; Levitzki & Gazit, 1995). Additional components of the signaling pathway that are of interest are the SH_3 and SH_2 domains, both of which are potential cancer targets. In the last four years several laboratories have reported on the application of combinatorial library methods to define the peptide binding motifs for SH_3 and SH_2 domains of several signaling proteins, and the substrate motif for the catalytic domain of PTKs. It is believed that, with the information obtained from these studies, one could design peptidomimetic drugs that can bind to these targets. Although there has been very little published work on screening small molecule combinatorial libraries for these cancer targets, many researchers are undoubtedly exploring that option in their laboratories at this time.

SH_3 domains

Two completely different combinatorial library methods have been used to elucidate the binding motifs for various SH_3 domains. Using the 'one-bead one-compound' library method, Yu *et al.* (1994) tagged the SH_3 domain of phosphatidylinositol-3-kinase (PI_3 kinase) or $p60^{c\text{-}src}$ PTK with a fluorescent probe and screened a semi-random peptide bead library XXXPPXPXX (wherein X = all 20 eukaryotic amino acids except cysteine), and isolated two classes of peptide motifs for each of the SH_3 domains. The binding affinity of several of the isolated ligands are between 9 and 70 μM. Some of these ligands exhibit selectivity towards one over the other SH_3 domain. For example, the PI_3 kinase SH_3 domain ligand, RKLPPRPSK, binds to the PI_3 kinase and Src SH_3 domains with a K_D of 9.1 and 52 μM respectively. More recently, the same group (Combs *et al.*, 1996) reported the discovery of peptide ligands with non-peptidic elements that bind to Src SH_3 domains, by screening a XYZ—PLPPLP library (wherein X, Y and Z are non-peptide components). The best ligands isolated in this screen had a K_D of 3.4 μM.

Cheadle *et al.* (1994) and Sparks *et al.* (1994), on the other hand, applied the filamentous phage displayed combinatorial peptide library method to screen for SH_3 binding motifs. In these experiments, the GST–SH_3 domain fusion protein was first immobilized on plastic and was then used to pan a filamentous phage displayed peptide library solution. Phages displaying proline-rich sequences were isolated. Sparks *et al.* (1994) also constructed a secondary phage library displaying the peptide X_6PXXPX_6, used it to identify ligands for eight different SH_3 domains and demonstrated that various SH_3 domains were able to discern subtle differences in the primary structure of the ligands.

SH_2 domains

Songyang *et al.* (1993) applied an affinity chromatography selection method to screen a synthetic combinatorial library. The semi-random peptide library, GDGPYXXXS (wherein X = all 20 eukaryotic amino acids except Trp and Cys), was synthesized using a 'split synthesis' approach. The completed library was then cleaved off the resin, precipitated with diethyl ether and applied on an affinity column of GST–SH_2 domain fusion proteins that had been immobilized on glutathione–agarose beads. The bound peptides were then eluted by 20 mM sodium phenylphosphate and microsequenced collectively without further purification. In this study the predominant motif identified for the one group of SH_2 domains (Src, Fyn, Lck, Fgr, Abl, Crt, and NcK) was pY–hydrophilic–hydrophilic–I/P while another group of SH_2 domains (p85, phospholipase C-γ and SHPTP2) was pY–hydrophobic–X–hydrophobic. Interestingly, the Src subfamily (Src, Fyn, Lck and Fgr) appears to prefer pYEEI. The affinity column selection approach appears to work well for peptide libraries as reported above. However, it is doubtful whether this approach can be applied to small organic libraries or oligomeric non-peptide libraries since structure determination of a mixture of these compounds would be almost impossible with the currently available methods.

Protein tyrosine kinase catalytic domain

Songyang *et al.* (1995) extended their affinity chromatography selection approach to discover peptide substrate motifs for various PTKs. In this method a semi-random peptide library, MAXXXYXXXXAKKKK (wherein, X = all 20 eukaryotic amino acids except Cys, Trp, Tyr, Ser and Thr), was synthesized, cleaved off the resin, ether precipitated and dissolved in buffer prior to mixing with ATP and PTK. The phosphorylated peptide solution was then applied to a ferric chelating column and the phosphorylated peptide solution eluted and microsequenced collectively. Many of the peptides isolated had a strong preference for glutamic acids. For example, the predominant peptide motif for $p60^{c\text{-}src}$ PTK was EEIYGEFF. A totally different approach was used in the author's laboratory to identify peptide substrates for $p60^{c\text{-}src}$ PTK (Lam *et al.*, 1995c). In this approach a random peptide bead library was mixed with [γ-^{32}P]ATP and $p60^{c\text{-}src}$ PTK. The ^{32}P-labeled peptide beads were then isolated for microsequencing (see Solid phase assay—functional assay). With this approach a discrete peptide, YIYGSFK (instead of a whole collection of peptides as in the case described by Songyang *et al.*), was identified as an efficient substrate for $p60^{c\text{-}src}$ PTK. Based on SAR studies, IY was then determined as an important motif, and a secondary library, XIYXXXX (wherein X = all 19 amino acids except Cys), was synthesized and screened as above. A new substrate, GIYWHHY, with a lower K_m value (~20 μM) was then isolated (Lou *et al.*, 1996). Pseudosubstrate-based peptide inhibitors developed from the GIYWHH template were designed with an IC_{50} value in the low micromolar range. These pseudo-substrate peptide inhibitors are highly specific (even within the Src-family PTK) and are excellent leads for developing peptidomimetic inhibitors for $p60^{c\text{-}src}$ PTK. Work is currently underway in the author's laboratory to screen small-molecule peptidomimetic libraries for $p60^{c\text{-}src}$ PTK substrates as well as inhibitors.

Random screening of the cell-based cytotoxic assay

Thus far, there have been few reports on applying combinatorial library methods to screen for cell-based cytotoxicity against intact tumor cells. Recently, an *in situ* releasable solution

phase assay to discover novel anti-cancer compounds has been reported (Salmon *et al.*, 1996). In this assay the compound bead libraries (whether peptides or small molecules) were synthesized on beads via cleavable linkers. The bead library was then mixed with a tumor cell line in 0.5% soft agar with tissue culture in a small Petri dish incubated at 37°C. Under neutral conditions, the 'diketopiperazine linker' is cleaved and the compound is released from the solid support and diffused outward from a bead. Some 24–48 h later 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added. In the presence of MTT, live cells turn purple, due to the accumulation of formazon, while dead cells remain colorless. As a result, a clear zone of inhibition occurs around compound beads with anti-cancer activity. The positive beads were then isolated for structure determination. With this approach, small tri- and tetrapeptides with unnatural amino acids and a non-peptide small molecule with anti-tumor activities were isolated (Salmon *et al.*, 1996).

Perspectives

Combinatorial library methods have been considered as one of the most important recent advances in medicinal chemistry and are currently being exploited by the pharmaceutical industry in the drug discovery effort. The field has advanced rapidly over the past five years. Efficient coupling chemistry on solid phase support and high throughput screening methods are being developed. Journals dedicated to this area have been started (e.g. *Molecular Diversity*). Publications in this field appear in virtually every issue of *Tetrahedron Letters*, *Journal of Medicine Chemistry*, *Journal of the American Chemical Society* and *Journal of Organic Chemistry*. Over 1000 articles on this area have been published in the last 6 years. Many new start-up companies in this area have emerged. In a matter of 6 years, promising drug leads have already been developed from using combinatorial library methods and several are currently in preclinical studies; it is anticipated that some of these leads will enter clinical trials in the very near future. In conjunction with the advent of computational chemistry and molecular modeling techniques, combinatorial chemistry can now be applied to the various new drug targets developed from our recent understanding of the molecular basis of cancer. The future of drug development for anti-cancer agents is bright and it is hopeful that more potent, more specific and less toxic agents will be developed in the foreseeable future. Lastly, I would like to emphasize that combinatorial chemistry, in addition to being a great tool for drug discovery, is also invaluable in basic research, which undoubtedly will provide a breakthrough in the understanding of cancer and in turn will facilitate the development of useful drugs for treating cancer patients.

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